

Detection of Experimental *Salmonella enteritidis* and *S. typhimurium* Infections in Laying Hens by Fluorescence Polarization Assay for Egg Yolk Antibodies

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ABSTRACT Identifying infected laying flocks is a critical component in efforts to prevent eggborne transmission of *Salmonella enteritidis* to humans. In the present study, egg yolk samples from experimentally infected chickens were tested for specific antibodies with a very rapid fluorescence polarization assay using tracers prepared from the O-polysaccharides of *S. enteritidis* and *S. typhimurium* and a conventional ELISA using an *S. enteritidis* flagellin antigen. In two trials, groups of specific-pathogen-free laying hens were infected orally with 10^6 or 10^8 cfu of *S. enteritidis* (phage type 13a) or with 10^8 cfu of *S. typhimurium*. Eggs were collected during five

weekly postinoculation intervals. Both fluorescence polarization and ELISA detected the majority of hens infected with *S. enteritidis* at either dose level, although they also frequently cross-reacted with samples from hens infected with *S. typhimurium*. Fluorescence polarization with an *S. typhimurium* tracer was likewise able to consistently detect *S. typhimurium* infection but also tended to cross-react with samples from hens infected with *S. enteritidis*. Fluorescence polarization appears to offer a simple and rapid alternative to conventional serological methodology, although concerns about specificity may limit the usefulness of antibody testing data.

(Key words: *Salmonella enteritidis*, fluorescence polarization, egg yolk antibodies)

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INTRODUCTION

Despite implementation of intensive disease-control efforts on several continents, the eggborne transmission of *Salmonella enteritidis* infection to humans remains a persistent and expensive international public health problem (Angulo and Swerdlow, 1999; Centers for Disease Control, 2000). Identifying infected commercial egg-laying flocks is a central objective of most risk reduction plans (Hogue et al., 1997). In the United States, a proposed national control program would screen flocks for *S. enteritidis* infection by bacteriological culturing of environmental samples and then confirm the existence of an ongoing threat to public health by culturing eggs (President's Council on Food Safety, 1999). A different strategy, based on testing for specific antibodies, was reported to detect *Salmonella* infections in Dutch and Danish breeder flocks at higher frequencies than did fecal (Feld et al., 2000) or environmental (Zijderveld et al., 1993) sampling. Enzyme immunoassay methods based on lipopolysaccharide or flagella antigens have been shown to detect infected poul-

try with high sensitivity, but lingering concerns about the ability of many serological tests to differentiate between *Salmonella* serotypes have limited their use (Barrow, 2000). Nevertheless, the potential speed and efficiency of serological methods continues to generate discussion about using them in *S. enteritidis* control programs.

Because eggs can be collected with minimal labor, without causing stress to laying hens or imperiling flock biosecurity, they are especially attractive samples for antibody testing. Antibodies to *S. enteritidis* have been found in the yolks of eggs laid by experimentally and naturally infected hens (Gast and Beard, 1991; Desmidt et al., 1996). Egg yolk antibody testing has consistently detected hens infected with oral doses as low as 10^3 cfu of *S. enteritidis* (Gast et al., 1997a). The presence of egg yolk antibodies has been shown to correlate with fecal shedding of (van de Giessen et al., 1992) and organ invasion by (Gast and Beard, 1991) *S. enteritidis*. Egg yolk antibody detection predicts *S. enteritidis* contamination of eggs by experimentally infected hens as effectively as does culturing fecal samples (Gast et al., 1997b).

Most antibody testing protocols have involved agglutination or ELISA formats. Fluorescence polarization (FP) technology offers an alternative with several advantages

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Abbreviation Key: FP = fluorescence polarization.

in comparison to more traditional methods (Rhys Williams, 1988; Nielsen et al., 1996). FP assays need only one liquid phase incubation step, requiring a single tracer reagent and less than 3 min to complete. FP testing is based on the inverse relationship between the size of molecules and their natural spin rate in a liquid medium (Nasir and Jolley, 1999). A beam of polarized light is used to determine the rate of spin of tracer molecules labeled with a fluorescent dye. Specific antibodies are detected when they bind to the tracer to produce a labeled antibody-tracer complex with a reduced rate of spin. In a recent study, an FP assay found antibodies to *S. enteritidis* in the sera of experimentally infected hens (Gast et al., 2002). The objective of the present study was to determine the sensitivity and specificity of FP tests using O-polysaccharide tracers for detecting egg yolk antibodies following experimental infection of chickens with *S. enteritidis* or *S. typhimurium*.

MATERIALS AND METHODS

Experimental Infection of Laying Hens

In each of two replicate trials, 36 Single Comb White Leghorn laying hens from our laboratory's specific-pathogen-free flock were housed individually in laying cages and provided ad libitum access to water and a pelleted, antibiotic-free diet (16.7% CP, 2,968 kcal ME/kg, 2.9% Ca, 0.39% P). The hens (hatchmates that were 26 and 32 wk old at the beginning of the first and second trials, respectively) were distributed into four groups in separate rooms of a disease-containment facility. One group of 10 hens in each trial was infected orally with a dose of 1.0×10^6 cfu of a phage type 13a strain of *S. enteritidis*, a second group of 10 hens was orally inoculated with a dose of 1.0×10^8 cfu of the same *S. enteritidis* strain, and a third group of 10 hens received an oral dose of 1.2×10^8 cfu of *S. typhimurium*. The remaining six hens in each trial served as an uninfected negative control group. Both *Salmonella* strains were prepared from frozen stocks by overnight incubation at 37 C in tryptone soya broth² and then diluted to obtain the desired cell density in each 1.0-mL dose.

Detection of Specific Egg Yolk Antibodies by Enzyme Immunoassay

Eggs were collected from each hen at five weekly post-inoculation (PI) intervals. Yolk samples from these eggs were diluted 1:250 in PBS and tested for the presence of specific antibodies by enzyme immunoassay (ELISA-SE) using purified *S. enteritidis* flagella (at 1 μ g/mL) as solid-phase detection antigens (Holt and Porter, 1993). Mono-

clonal antibodies specific for chicken IgG (prepared by the authors and used at a 1:40 dilution) and alkaline phosphatase-labeled goat anti-mouse IgG³ (used at a 1:750 dilution) were added in sequence to facilitate colorimetric detection. After adding *p*-nitrophenol phosphate as a substrate, color development was evaluated by determining the absorbance at 405 nm. Egg yolk samples were considered to be antibody-positive in this test if their ELISA absorbance values exceeded the mean absorbance value for the negative control samples (taken from the uninfected group) by more than two standard deviations.

Detection of Specific Egg Yolk Antibodies by FP

Egg yolks were also tested for specific antibodies using three FP assays (Nielsen et al., 1996). Each sample was diluted 1:50 in PBS, and a baseline FP value was determined in a Sentry reader.⁴ After this blanking step, 10 μ L of tracer⁴ (a fluorescein-labeled preparation of *Salmonella* O-polysaccharide purified using a polymyxin B column) was added to the sample, and the FP reading was taken again after 2 min. Each egg yolk was tested with two *S. enteritidis* tracers prepared under slightly different conditions (FP-SE1 and FP-SE2) and an *S. typhimurium* tracer (FP-ST). Egg yolk samples were considered to be antibody-positive in these tests if their FP values exceeded the mean FP value for the negative control samples (taken from the uninfected group) by more than two standard deviations.

Statistical Analyses

Significant differences ($P < 0.05$) between treatment groups or between tests in the frequency of identification of hens as antibody-positive were determined by applying Fisher's exact test to data organized into 2×2 contingency tables using Instat biostatistics software.⁵ As no significant variation was observed between the two replicate trials, the results were combined for analysis.

RESULTS

No hens from the uninfected negative control group were identified as antibody-positive by any test. When egg yolks from hens inoculated with a dose of 10^6 cfu of *S. enteritidis* were tested by ELISA-SE, 24% of the samples were antibody-positive at 1 wk PI, 47% at 2 wk PI, and 76% at 4 wk PI (Figure 1a). The FP tests with *S. enteritidis* tracers yielded positive results more slowly but eventually attained peak detection levels of 82% (FP-SE1 at 4 wk PI) and 69% (FP-SE2 at 5 wk PI). Overall, for all sampling dates combined, a significantly ($P < 0.01$) lower frequency of positive results was obtained by FP-SE2 than by ELISA-SE. The FP-ST assay cross-reacted with as many as 59% (at 4 wk PI) of egg yolk samples from these hens.

After inoculation of hens with 10^8 cfu of *S. enteritidis* (Figure 1b), ELISA-SE detected antibodies in 44% of egg yolk samples at 1 wk PI, 67% at 2 wk PI, and 95% at 3

²Unipath Co., Oxoid Division, Ogdensburg, NY.

³Calbiochem Corp., La Jolla, CA.

⁴Diachemix Corp., Grayslake, IL.

⁵GraphPad Software, San Diego, CA.

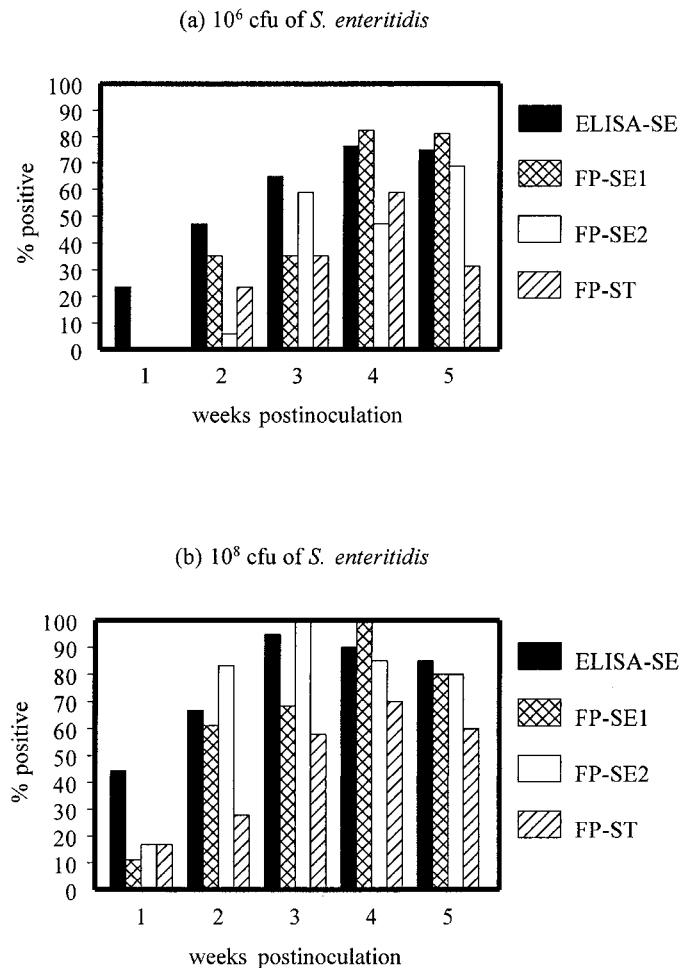


FIGURE 1. Detection of antibodies in laying hens infected orally with either 10⁶ (a) or 10⁸ (b) CFU of *S. enteritidis* (SE). Serum samples were tested with an enzyme immunoassay (ELISA) using an *S. enteritidis* flagella antigen and fluorescence polarization (FP) assays using O-polysaccharide tracers from *S. enteritidis* (two different tracers designated SE1 and SE2) and *S. typhimurium* (ST).

wk PI. The FP assays all gave relatively few positive results at 1 wk PI, but FP-SE2 and FP-SE1 identified 100% of samples as antibody-positive by 3 and 4 wk PI, respectively. For all sampling dates combined, no significant differences were observed between the frequencies of antibody detection by the three *S. enteritidis* tests. Significantly fewer ($P < 0.02$) positive results were obtained with the FP-ST assay, although cross-reactions were observed with as many as 70% of these samples (at 4 wk PI).

When applied to egg yolks from laying hens infected with 10⁸ cfu of *S. typhimurium*, the FP-ST assay detected antibodies in 88% of these samples by 5 wk PI (Figure 2). For all sampling intervals combined, FP-ST provided significantly ($P < 0.001$) more positive results than either of the FP tests with *S. enteritidis* tracers, although cross-reactions were observed with as many as 67% of these samples using the FP-SE1 assay (at 4 wk PI) and 44% (at 3 wk PI) using the FP-SE2 assay. Significantly ($P < 0.02$) more of these samples were identified as antibody-positive by ELISA-SE (up to a peak level of 75% at 3 wk PI) than by either FP test for *S. enteritidis*.

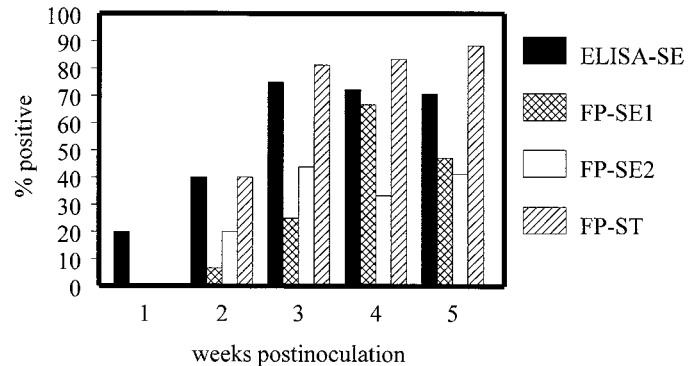


FIGURE 2. Detection of antibodies in laying hens infected orally with 10⁸ cfu of *S. typhimurium*. Serum samples were tested with an enzyme immunoassay (ELISA) using an *S. enteritidis* flagella antigen and fluorescence polarization (FP) assays using O-polysaccharide tracers from *S. enteritidis* (two different tracers designated 1 and 2) and *S. typhimurium* (ST).

DISCUSSION

In the present study, *S. enteritidis* and *S. typhimurium* infections in laying hens were detected at high frequencies by testing for egg yolk antibodies with FP assays using homologous O-polysaccharide tracers. Antibodies to *S. enteritidis* have also been previously detected with high efficiency by FP in the sera of experimentally infected chickens (Gast et al., 2002). At most sampling intervals after 1 wk PI, antibodies to *S. enteritidis* were found in egg yolks at similar frequencies by FP and ELISA. In prior studies, egg yolk antibodies have not typically been reported to be present at high titers during the first week following oral inoculation (Gast and Beard, 1991; Sunwoo et al., 1996; Gast et al., 1997b). In several earlier investigations, the detection of specific antibodies in chickens has correlated positively with the isolation of *S. enteritidis* from feces, tissues, and eggs (Gast and Beard, 1991; van de Giessen et al., 1992; Gast et al., 1997b; Gast and Holt, 2001). Commercial laying flocks have been identified as infected by enzyme immunoassays using diverse preparations containing *S. enteritidis* flagella (McDonough et al., 1998; Zamora et al., 1999). The flagellar ELISA used in the present experiments was shown to be highly sensitive in an international collaborative study (Barrow et al., 1996). Accordingly, FP assays may offer a rapid and effective alternative for serological screening of laying flocks for *S. enteritidis* infection.

None of the assays used in the present study demonstrated an especially high degree of specificity for differentiating *S. enteritidis* and *S. typhimurium* infections. No clear or consistent pattern of sensitivity and specificity distinguished the two *S. enteritidis* FP tracers. The flagellar ELISA was previously characterized as highly specific in comparison to a wide assortment of other serological tests (Barrow et al., 1996). The high oral doses of *Salmonella* used in the present trials likely posed a very severe challenge to the capabilities of the various assays to specifically detect infections with individual serotypes. Polysaccharide and flagella antigens of salmonellae are distrib-

uted across serotype and serogroup lines (Nicholas and Cullen, 1991; Barrow, 2000), often complicating the interpretation of serological testing results. In a large recent field study of commercial laying flocks in the United States, the detection of egg yolk antibodies by an ELISA using a fimbrial antigen of presumed high specificity did not correlate with the isolation of *S. enteritidis* from environmental samples (USDA, 2000). Because antibody tests for *S. enteritidis* are currently of interest mainly for identifying potentially infected flocks as a prelude to intensive egg culturing to isolate the pathogen, specificity is arguably a less critical assay characteristic than is sensitivity. Nevertheless, inadequate specificity of serological screening tests could cause resources to be wasted when subsequent egg culturing is conducted in flocks infected with *Salmonella* serotypes that are antigenically cross-reactive but of no known public health significance.

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REFERENCES

- Angulo, F. J., and D. L. Swerdlow. 1999. Epidemiology of human *Salmonella enterica* serovar Enteritidis infections in the United States. Pages 33–41 in *Salmonella enterica* serovar Enteritidis in Humans and Animals. A. M. Saeed, R. K. Gast, M. E. Potter, and P. G. Wall, ed. Iowa State University Press, Ames, IA.
- Barrow, P. A. 2000. Serological diagnosis of *Salmonella* by ELISA and other tests. Pages 407–427 in *Salmonella in Domestic Animals*. C. Wray and A. Wray, ed. CABI Publishing, Oxon, UK.
- Barrow, P. A., M. Desmidt, R. Ducatelle, M. Guittet, H. M. J. F. van der Heijden, P. S. Holt, J. H. J. Huis in't Velt, P. McDonough, K. V. Nagaraja, R. E. Porter, K. Proux, F. Sisak, C. Staak, G. Steinbach, C. J. Thorns, C. Wray, and F. van Zijderfeld. 1996. World Health Organisation-supervised interlaboratory comparison of ELISAs for the serological detection of *Salmonella enterica* serotype Enteritidis in chickens. *Epidemiol. Infect.* 117:69–77.
- Centers for Disease Control. 2000. Outbreaks of *Salmonella* serotype Enteritidis infection associated with eating raw or undercooked shell eggs—United States, 1996–1998. *Morbidity and Mortality Weekly Rep.* 49:73–79.
- Desmidt, M., R. Ducatelle, F. Haesebrouck, P. A. De Groot, M. Verlinden, R. Wijffels, M. Hinton, J. A. Bale, and V. M. Allen. 1996. Detection of antibodies to *Salmonella enteritidis* in sera and yolks from experimentally and naturally infected chickens. *Vet. Rec.* 138:223–226.
- Feld, N. C., L. Ekeroth, K. O. Gradel, S. Kabell, and M. Madsen. 2000. Evaluation of a serological *Salmonella* Mix-ELISA for poultry used in a national surveillance programme. *Epidemiol. Infect.* 125:263–268.
- Gast, R. K., and C. W. Beard. 1991. Detection of *Salmonella* serogroup D-specific antibodies in the yolks of eggs laid by hens infected with *Salmonella enteritidis*. *Poult. Sci.* 70:1273–1276.
- Gast, R. K., and P. S. Holt. 2001. The relationship between the magnitude of the specific antibody response to experimental *Salmonella enteritidis* infection in laying hens and their production of contaminated eggs. *Avian Dis.* 45:425–431.
- Gast, R. K., M. S. Nasir, M. E. Jolley, P. S. Holt, and H. D. Stone. 2002. Serological detection of experimental *Salmonella enteritidis* infections in laying hens by fluorescence polarization and enzyme immunoassay. *Avian Dis.* 46:137–142.
- Gast, R. K., R. E. Porter, Jr., and P. S. Holt. 1997a. Assessing the sensitivity of egg yolk antibody testing for detecting *Salmonella enteritidis* infections in laying hens. *Poult. Sci.* 76:798–801.
- Gast, R. K., R. E. Porter, Jr., and P. S. Holt. 1997b. Applying tests for specific yolk antibodies to predict contamination by *Salmonella enteritidis* in eggs from experimentally infected laying hens. *Avian Dis.* 41:195–202.
- Hogue, A., P. White, J. Guard-Petter, W. Schlosser, R. Gast, E. Ebel, J. Farrar, T. Gomez, J. Madden, M. Madison, A. M. McNamara, R. Morales, D. Parham, P. Sparling, W. Sutherland, and D. Swerdlow. 1997. Epidemiology and control of egg-associated *Salmonella* Enteritidis in the United States of America. *Rev. Sci. Tech. Off. Int. Epiz.* 16:542–553.
- Holt, P. S., and R. E. Porter, Jr. 1993. Effect of induced molting on the recurrence of a previous *Salmonella enteritidis* infection. *Poult. Sci.* 72:2069–1078.
- McDonough, P. L., R. H. Jacobson, J. F. Timoney, A. Mutalib, D. C. Kradel, Y.-F. Chang, S. J. Shin, D. H. Lein, S. Trock, and K. Wheeler. 1998. Interpretations of antibody responses to *Salmonella enterica* serotype Enteritidis gm flagellin in poultry flocks are enhanced by a kinetics-based enzyme linked immunosorbent assay. *Clin. Diagn. Lab. Immunol.* 5:550–555.
- Nasir, M. S., and M. E. Jolley. 1999. Fluorescence polarization: an analytical tool for immunoassay and drug discovery. *Combinatorial Chem. High Throughput Screening* 2:177–190.
- Nicholas, R. A. J., and G. A. Cullen. 1991. Development and application of an ELISA for detecting antibodies to *Salmonella enteritidis* in chickens flocks. *Vet. Rec.* 128:74–76.
- Nielsen, K., D. Gall, M. Jolley, G. Leishman, S. Balsevicius, P. Smith, P. Nicoletti, and F. Thomas. 1996. A homogeneous fluorescence polarization assay for detection of antibody to *Brucella abortus*. *J. Immunol. Methods* 195:161–168.
- President's Council on Food Safety. 1999. Egg safety from production to consumption: an action plan to eliminate *Salmonella enteritidis* illnesses due to eggs. President's Council on Food Safety, Washington, DC.
- Rhys Williams, A. T. 1988. Fluorescence polarization immunoassay. Pages 135–147 in *Complementary immunoassays*. A. T. Rhys Williams, ed. John Wiley, Chichester, UK.
- Sunwoo, H. H., T. Nakano, W. T. Dixon, and J. S. Sim. 1996. Immune responses in chickens against lipopolysaccharide of *Escherichia coli* and *Salmonella typhimurium*. *Poult. Sci.* 75:342–345.
- USDA. 2000. *Salmonella enterica* serotype Enteritidis in table egg layers in the U.S. USDA, Fort Collins, CO.
- van de Giessen, A. W., J. B. Dufrenne, W. S. Ritmeester, P. A. T. A. Berkers, W. J. van Leeuwen, and S. H. W. Notermans. 1992. The identification of *Salmonella enteritidis*-infected poultry flocks associated with an outbreak of human salmonellosis. *Epidemiol. Infect.* 109:405–411.
- Zamora, B. M., M. Hartung, G. Hildebrandt and A. Ksbohrer. 1999. Detection of antibodies to *S. enteritidis* in broilers by means of indirect ELISA and chemiluminescent immunoassay (CLIA). *J. Vet. Med B* 46:9–23.
- Zijderfeld, F. G. van, A. M. van Zijderfeld-van Bemel, R. A. M. Brouwers, T. S. de Vries, W. J. M. Landman, and W. A. de Jong. 1993. Serological detection of chicken flocks naturally infected with *Salmonella enteritidis*, using an enzyme-linked immunosorbent assay based on monoclonal antibodies against the flagellar antigen. *Vet. Q.* 15:135–137.